

Morphological changes induced by neuropeptide in vitro stimulation of the rat parotid gland

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Summary

The effect of in vitro stimulation of rat parotid gland with the neuropeptides substance P, calcitonin gene-related peptide and galanin has been studied by microfilament fluorescence staining and in semithin sections, and compared to control incubations and in vitro stimulation with β -adrenergic and muscarinic agonists. Clear-cut aspects of massive granule exocytosis and cytoplasm vacuolation, indicative of protein and fluid secretion respectively, were obvious only after substance P stimulation, whereas treatment with galanin and calcitonin gene-related peptide produced little to no morphological changes. The results being in agreement with the outcome of other methodological approaches, these procedures appear reliable, may be effectively applied to the study of the functional regulation of secretory mechanisms, and may be particularly useful in human tissue analyses.

Key words

Substance P, calcitonin gene-related peptide, galanin, immunohistochemistry, F-actin staining, semithin sections

Introduction

Saliva is a watery solution of electrolytes, small organic substances and a variety of proteins and glycoproteins with enzymatic, lubricating, defensive and protective functions. Salivary secretion is controlled by the synergistic activity of the two autonomic nervous system sections. The stimulation of parasympathetic muscarinic receptors causes a copious secretion of water and electrolytes, via Ca^{2+} mobilization from intracellular stores, whereas the stimulation of sympathetic β -adrenergic receptors produces a small amount of secretion rich in exocytosed proteins, via c-AMP as second messenger (Schramm and Selinger, 1975). Morphological studies on rat salivary glands show that a dramatic sequential reorganization of the luminal acinar cell membrane accompanies exocytosis (Amsterdam et al., 1969) and that stimulation of either β -adrenergic or muscarinic receptors induces two distinct exocytosis-coupled-to-endocytosis mechanisms (Segawa and Riva, 1996). In fact, β -adrenergic agonists, together with extensive degranulation of acinar cells, cause the enlargement of the luminal space and the appearance, along the apical membrane, of many Ω -shaped contours enwrapped by microfilaments, whose size is similar to that of the secretory granules (Segawa et al., 2000). By contrast, muscarinic agonists elicit the growth of cytoplasmic vacuoles, whose membrane is not surrounded by microfilaments, con-

nected to the luminal membrane and space (Segawa *et al.*, 2000). On the other hand, besides the classical cholinergic and adrenergic neuromediators, numerous peptides, collectively designated as non-adrenergic non-cholinergic (NANC) agonists, are known to participate with different effectiveness in the secretory mechanisms and hence influence the composition of saliva (Gallacher, 1983; Ekström, 1989). Among them, substance P (SP) has a potent sialogogic activity in some species, including rat (Lembeck and Starke, 1968; Ekström, 1987), a property that has been exploited as a bioassay to obtain its purification (Leeman and Hammerschlag, 1967; Chang and Leeman, 1970), whereas calcitonin gene-related peptide (CGRP) may affect the activity of other sialogogic peptides, but causes no secretion on its own (Ekström, 1987; Ekström *et al.*, 1988), and galanin (GAL) may be functionally relevant in some rat salivary glands (Konopka *et al.*, 2004; Szőke *et al.*, 2009).

In this study, the morphological changes consequent to the *in vitro* stimulation of the rat parotid gland with the neuropeptides SP, CGRP and GAL have been examined and compared to those induced by β -adrenergic and muscarinic agonists. The occurrence of those peptides in the gland was also checked by immunohistochemistry.

Materials and methods

Parotid glands of 10-15 week old male Wistar rats were used.

Immunohistochemistry

Three rats were transcardially perfused with phosphate-buffered 4% paraformaldehyde, pH 7.3, and the glands excised, postfixed in the same fixative for 2 hours and rinsed overnight in 0.1 M phosphate buffer, pH 7.3. Indirect immunofluorescence was elicited in 10-14 μm thick cryostat sections placed on coated slides, using as primary antiserum either a rat monoclonal antibody against SP (gift of Dr A.C. Cuello, diluted 1:50), a rabbit antiserum against CGRP (Peninsula, diluted 1:300), or a rabbit antiserum against GAL (CRB, diluted 1:1200). After overnight incubation and rinsing, the sections were incubated for 1 hour at room temperature with FITC-conjugated anti-rat or anti-rabbit secondary antisera, as appropriate. All antisera were diluted in phosphate buffered saline (PBS) containing 0.2% Triton X-100. Slides were mounted in glycerol/PBS 3:1 (v/v).

In vitro stimulation

Glands were rapidly excised from 3 rats and cut in small pieces. Samples were incubated at 37°C, for 45 min in constant agitation, in Dulbecco's modified Eagles minimum medium (Segawa *et al.*, 1985) added with either 20 μM D,L-isoproterenol (IPR), 100 μM carbachol (CCh), 10⁻⁶ μM SP, 10⁻⁶ μM CGRP and 10⁻⁶ μM GAL, or without chemicals as controls. For microfilament F-actin staining, samples were fixed with phosphate-buffered 4% paraformaldehyde, pH 7.3, for 1 hour, rinsed in 0.1 M phosphate buffer, pH 7.3, cryostat cut in sections of 10-14 μm and stained with TRITC-conjugated phalloidin (Molecular Probes, OR). For bright-field analysis, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, for 1 hour at room

temperature, postfixed in 1% OsO₄ at 4 °C, for 1 hour, dehydrated and embedded in epoxy resin. Semithin sections were stained with toluidine blue.

Slides were observed with a Leitz Dialux 20 microscope equipped with epifluorescence optics.

Results

Nerve fibres immunoreactive to SP, CGRP and GAL were found mainly around blood vessels and ducts, but also scattered in close proximity to acinar cells, with higher frequency for SP than for CGRP and GAL (Fig. 1A-E).

Analysis of granule exocytosis by microfilament F-actin staining showed that, compared to control incubations, where plasmalemmal contours appeared smooth and intercellular canaliculi and acinar lumen narrow and regular (Fig. 1F), in vitro stimulation with IPR caused the appearance of many Ω -shaped profiles along the luminal cell membrane and dilation of the acinar lumen (Fig. 1G), whereas CCh stimulation did not change the aspect detectable in controls (Fig. 1H). Treatment with SP resulted in widespread presence of numerous Ω -shaped profiles crowded towards a dilated lumen in many acini (Fig. 1I), whereas aspects of modifications of the luminal membrane were rare after treatment with GAL (Fig. 1J) and absent after treatment with CGRP (Fig. 1K).

In toluidine blue-stained semithin sections of control specimens the acinar cells contained numerous secretion granules distributed in a wide area of the apical cytoplasm and the acinar lumen and interacinar space were narrow (Fig. 1L). Specimens stimulated in vitro with IPR showed conspicuous granule exocytosis, apicalization of the remaining secretory granules and enlargement of the luminal and interacinar space (Fig. 1M), and CCh treated samples showed many vacuoles in the acinar cell cytoplasm (Fig. 1N). Stimulation with SP resulted in dramatic degranulation and apicalization of the remaining secretory granules accompanied by marked luminal space swelling, as well as in cytoplasm vacuolization (Fig. 1O). GAL treatment produced only a moderate degree of granule apicalization with sporadic and modest enlargement of the luminal space (Fig. 1P). Incubation with CGRP had no marked effects on granule density and distribution (Fig. 1Q).

Discussion

Our results on the occurrence and distribution of SP- and CGRP-positive innervation of the rat parotid gland agree with previous data (Sharkey and Templeton, 1984; Ekström, 1987; Ekström et al., 1988), whereas, to our knowledge, GAL-positive nerve fibres, though reported in other rat salivary glands (Konopka et al., 2004), have not been described in this organ previously. The source of SP innervation is largely parasympathetic, whereas a substantial amount of the CGRP-containing fibres also has a sensory origin (Sharkey and Templeton, 1984; Ekström et al., 1988). The morphological changes ensuing from SP in vitro stimulation appear in agreement with data showing that in the presence of autonomic blockade SP evokes amylase release and fluid secretion from rat parotid (Gallacher, 1983) and that parasympathetic stimula-

tion not only evokes a copious watery secretion, but also causes protein release and granule exocytosis via NANC mediators (Ekström *et al.*, 1998; Ekström, 2002).

In a similar way, the outcome after CGRP stimulation is in agreement with the fact that, contrary to SP, this peptide does not evoke any flow of saliva after intravenous injection and is little effective in evoking amylase release *in vitro* (Ekström *et al.*, 1988). As for GAL, a major effect on the rat parotid has not been detected, though its functional relevance can not be excluded.

In conclusion, this morphological approach appears as a simple and reliable way to contribute effectively to functional studies on the regulation of gland activity and secretory mechanisms. Considering the high interspecific variability, which advises against the extrapolation to man of data obtained in experimental animals, it may be particularly useful when studying human tissues, as it may overcome the hindrance to systemic *in vivo* treatments.

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Figures

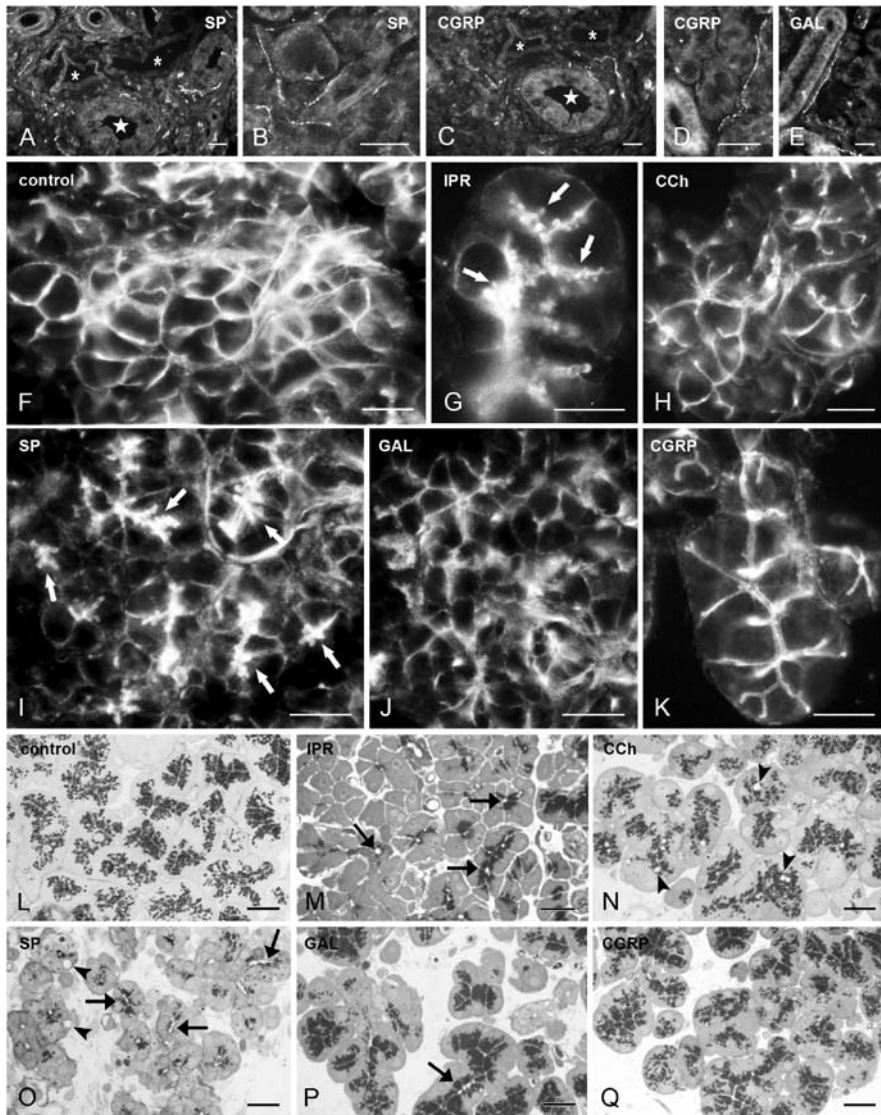


Fig. 1 – A-E: Nerve fibres immunoreactive to SP (A,B), CGRP (C,D) and GAL (E) in perivasular, periductal and periacinar position; A and C are semiconsecutive sections: the two asterisks label the same blood vessels and the star labels the same duct. F-K: microfilament fluorescence staining of parotid acini in vitro incubated as control (F) and with IPR (G), CCh (H), SP (I), GAL (J), and CGRP (K); arrows in G and I point to obvious aspects of granule exocytosis. L-Q: toluidine-blue stained semithin sections of samples in vitro incubated as control (L) and with IPR (M), CCh (N), SP (O), GAL (P), and CGRP (Q); acinar degranulation is remarkable in M and O; arrows in M, O, P point to apical granules and luminal space swellings; arrowheads in N and O point to sites of vacuolization. Scale bars: A-D = 50 μ m; E = 25 μ m; F-Q = 20 μ m.